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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 3/18 — A1

(11) International Publication Number: WO 90/08158

(43) International Publication Date: 26 July 1990 (26.07.90)

(21) International Application Number: PCT/DK90/00016

(22) International Filing Date: 18 January 1990 (18.01.90)

(30) Priority data:
4123/89 19 January 1989 (19.01.89) DK

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: METHOD OF RECOVERING PURIFIED EPI PROTEIN FROM A SOLUTION ESPECIALLY A FERMENTATION SOLUTION

(57) Abstract

The EPI protein is isolated and purified from a fermentation solution, using chromatographic technique, wherein the solution containing the EPI protein is applied to a matrix coupled with heparin, preferably heparin-Sepharose.

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Method of recovering purified EPI protein from a solution especially a fermentation solution.

The present invention refers to a method of isolating and purifying the EPI protein.

Background of the invention

Blood coagulation is a complex process involving many activating and inactivating coagulation factors. Anticoagulant proteins are known to be important for regulation of the coagulation process. See B. Lämmle and J. Griffin (Clinics in Haemotolog 14, p. 281-342, 1985) for a review on coagulation inhibitors and regulation of coagulation.

Thus heparin is used clinically to increase the activity of antithrombin III and heparin cofactor II. Antithrombin III is used for the inhibition of factor Xa and thrombin. Hirudin is used for the inhibition of thrombin. Protein C may be used for the inhibition of factors V and VIII.

Coagulation can be initiated through the extrinsic pathway by the release of tissue factor (J.H. Morrissey et al.: Thromb Res 50, p. 481-93, 1988). Coagulation activation by the extrinsic pathway may be inhibited by different mechanisms (P.M. Sandset et al.: Thromb Res 47, p. 389-400, 1987; B.J. Warn-Cramer et al.: Thromb Res 48, p. 11-22, 1987; S. Kondo and W. Kisiel: Blood 70, p. 1947-54, 1987; S.D. Carson: J. Biol Chem 262, p. 718-21, 1987; G.J. Broze et al.: Blood 77, p. 335-43, 1988; S. Kondo et al.: Thromb Res 48, p. 449-59, 1987).

The basic trigger in many coagulation disorders is the release of tissue factor and thus activation of factor X by factor VII-tissue factor. During surgery, tissue factor is released

and thrombi may be formed. In heart attack a primary thrombus is formed and when this thrombus is released, tissue factor is exposed and coagulation is initiated resulting in a secondary, perhaps lethal thrombus. During sepsis, bacterial endotoxin induces the systemic release of tissue factor. This may lead to disseminated intravascular coagulation (DIC). DIC can be treated with antithrombin III (T.E. Emerson et al.: Circulatory Shock 21, p. 1-13, 1987) which inhibits the late steps in the coagulation cascade. Activated Protein C which inhibits in the middle of the coagulation cascade can also be used for the treatment of DIC (F.B. Taylor et al.: J Clin Invest 79, p. 918-25, 1987).

Protein showing extrinsic coagulation Pathway Inhibitor (EPI) activity has been recovered and isolated from human cells. It is known that EPI inhibits factor VII-tissue factor catalyzed activation of FX. However, the exact mechanism by which EPI inhibits coagulation is not known. Human plasma contains 3 molecular species showing EPI activity. The molecular masses are > 500 kDa, 200 kDa and 40 kDa respectively (P.M. Sandset et al.: Thromb Res 47, p. 389-400, 1987).

The object of the present invention is an improved method to isolate the protein EPI in concentrated or pure form.

Detailed description of the invention

Definitions: EPI is Extrinsic coagulation Pathway Inhibitor. EPI is a protein which shows activity in the assay described by Sandset et al. (Throm Res 47, p. 389-400, 1987). One unit of EPI is the amount of EPI activity found i 1 ml of normal human plasma.

The EPI protein may be recovered from supernatants of cell lines using precipitation and affinity chromatography on factor Xa. However, this purification procedure cannot be used

in large scale owing to the very limited availability of factor Xa (G.J. Broze and J.P. Miletich, Proc Natl Acad Sci USA 84, p. 1886-1890, 1987).

It has now been found that a very efficient purification is obtained when the solution containing the EPI protein is applied to a matrix coupled with heparin.

The invention is based on the discovery that the EPI molecule contains a specific site having affinity to heparin. A column containing a matrix coupled with heparin will selectively bind the EPI protein.

A preferred matrix is heparin-Sepharose, from which the EPI protein may be eluted.

Thus it is possible to apply 100 vol. culture medium on 1 vol. heparin-Sepharose and 80% of the EPI activity will be found in the eluate.

On the other hand if an- or cation exchange is used the result will be poorer. 25 vol. of protein free culture medium applied on 1 vol. Q-fast flow Sepharose will only give 10% EPI activity in the eluate and 35 vol. of protein free culture medium applied on 1 vol. S-fast flow Sepharose will only give 1% EPI activity in the eluate.

It is also possible to use precipitation with $CdCl_2$ as a first step for purification of EPI but this will only give a specific activity of 8.5 U/mg (G.J. Broze and J.P. Miletich: Proc. Natl Acad Sci USA 84, p. 1886-1890, 1987).

The invention is illustrated in the following with reference to the examples.

EXAMPLE 1

The human HeLa cell line was grown to confluency in Dulbecco-modified Eagles medium (DMEM) containing 10% fetal calf serum. The cell layer was washed free of serum proteins with serum-free DMEM and incubated in this medium at 37°C in an atmosphere of 5% CO_2. The medium was replaced and the spent medium frozen every two or three days.

Serum-free DMEM medium was harvested from the HeLa cell line as described above. 550 ml medium was applied over night at 4°C on a column containing 4 ml heparin-Sepharose equilibrated with buffer A (20 mM tris, 10% glycerol, 0.1 M NaCl, pH 7.5). The column was washed with 60 ml A and eluted with a gradient of 120 ml. A → B. Buffer B was 20 mM tris, 10% glycerol, o.7 M NaCl, pH 7.5. Fractions were collected and a pool was prepared from the fractions with highest activity. The pool contained 153 units of EPI for each mg of protein. 4 ml of the heparin Eluate was further fractionated on a microbore® RP4 column equilibrated in buffer C (0.1% TFA). EPI was eluted with a gradient from 25 to 60% of buffer D (60% isopropanol, 0.08% TFA) in buffer C. Elution was over 20 min with a flow of 0.15 ml/min. EPI eluted with a specific activity of 3600 units/mg protein (Table 1).

Table 1

Purification of EPI protein from serum-free HeLa culture medium

Fraction	EPI activity U/ml	Volume ml	Yield from culture med.	Spec. act. U/mg	Spec. act. rel. to human pl.
Culture medium	0.56	550	100	5.6[a]	400
Heparin-Seph.el.	5.4	35	61	153[b]	10,900
RP4 el.	25.2	0.35	25	3600[b]	260,000

a: Serum-free culture medium contained 0.1 mg protein ml

b: E_{280} (1%) was set to 10.

EXAMPLE 2

Serum-free DMEM medium was harvested from the HeLa cell line as described in Example 1. 600 ml medium was applied over night on a column containing 5.5 ml heparin-Sepharose equilibrated with buffer A (20 mM tris, 10% glycerol, 0.1M NaCl, pH 7.5). The column was washed with 20 ml buffer A and 80 ml buffer B (20 mM tris, 10% glycerol, 0.2 M NaCl, pH 7.5). The column was eluted with buffer C (20 mM Tris, 10% glycerol, 0.7 M NaCl, pH 7.5). 12 ml eluate containing EPI was pooled. 8 ml of the pool was diluted to 80 ml with buffer D (20 mM tris, 10% glycerol, pH 7.49. 75 ml was applied over 150 minutes on a mono-Q column equilibrated with buffer D. EPI was eluted with a gradient from 0 to 100% of buffer E (20 mM tris, 10% glycerol 0.5 M NaCl, pH. 7.4). Elution was over 45 min with a flow of 0.5 ml/min. EPI eluted in 3 ml, with a specific activity of 1030 units/mg in the top fractions (Table

2).

Table 2

Purification of EPI proteins from serum-free HeLa culture medium.

Fraction	EPI activity U/ml	Volume ml	Yield from culture med.	Spec. act. U/mg	Spc. act. rel. to human pl.
Culture med.	0.46	600	100	4.6[a]	326
Heparin-Sehp. eluate (step)	18.0	12	78	58[b]	4,100
Mono Q eluate	38.2	3	66	450[b]	32,100
MonoQ eluate top fraction	49.5	1.5	43	1030[b]	73,570

a: Serum-free culture medium contained 0.1 mg protein/ml

b: E_{280} (1%) was set to 10

EXAMPLE 3

EPI from serum-free DMEM medium was purified using Heparin-Sepharose and Mono-Q as described in Example 2. 75 units of Mono-Q EPI eluate were fractionated on a microbore RP-4 column (Browmlee 2 x 30 mm). The column was equilibrated in 75% buffer A (0.1% TFA) and 25% buffer B (0.08% TFA, 60% isopropanol). EPI was in 20 minutes eluted with 24 → 60% buffer B at a flow rate of 0.15 ml/min. 54% of the applied EPI activity was recovered in the eluate. 17% of the applied activity was recovered in a fraction that only, apart from gel artefacts, contained one protein as detected from SDS-PAGE. The fraction contained 23.000 units of EPI activity for each

mg of protein. The protein component was analyzed by measuring E_{280} (E280, 1% = 10) using Applied Biosystems photometer. The molecular weight in reduced SDS-PAGE is estimated to be 43 kDa, N-terminal sequencing on an Applied Biosystems gasphase sequenator gave the following sequence: Asp Ser Glu Glu Asp Glu Glu His Thr Ile Ile Thr X X Glu Leu Pro, where the amino acids at X could not be identified.

EXAMPLE 4

EPI from serum-free DMEM medium was purified using Heparin-Sepharose and MonoQ as described in Example 3. 4 ml of Mono-Q eluate was diluted with 15 ml of buffer A (20 mM Na_3-citrate, 10% glycerol, pH 5). The diluted sample was applied with a flow of 0.4 ml/min on a 1 ml Mono-S column equilibrated in buffer A. The column was washed with 10 ml of A and eluted in 50 minutes with a gradient of A → B (50 mM imidazol, 10% glycerol, 0.6 M NaCl, pH 7.47). The flow was o.5 ml/min. 58% of the applied amount of EPI was recovered with specific activity of 12.000 units/mg. The protein content was analyzed by measuring E_{280} (E280, 1% = 10) using a Pharmacia photometer.

EPI appears as a protein with a molecular weight of 43 kDa.

PATENT CLAIMS:

1. In a method of isolating and purifying an EPI protein from a solution using chromatographic technique, the improvement that the solution containing the EPI is applied to a matrix coupled with heparin.

2. Method according to claim 1, wherein the matrix is heparin-Sepharose.

3. Method according to claim 1 and 2, wherein the solution is a fermentation solution.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 90/00016

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 3/18

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 07 K; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A, 4721572 (R.E. JORDAN) 26 January 1988, see column 1, line 35 - column 2, line 40 --	1-3
A	Thrombosis Research, Vol. 48, 1987 B.J. Warn-Cramer et al.: "Partial purification and characterization of extrinsic pathway inhibitor (the factor Xa-dependent plasma inhibitor of factor VIII a/tissue factor) ", see page 11 - page 22 --	1-3
A	Proc. Natl. Acad. Sci., Vol. 84, April 1987 G.J. Broze et al.: "Isolation of the tissue factor produced by HepG2 hepatoma cells ", see page 1886 - page 90 --	1-3

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th April 1990	1990 -04- 20
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Niklas Forslund

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Thrombosis Research, Vol. 47, 1987 P.M. Sandset et al.: "A sensitive assay of extrinsic coagulation pathway inhibitor (EPI) in plasma and plasma fractions ", see page 389 - page 400 --	1-3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 90/00016

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4721572	88-01-26	EP-A- 0208215	87-01-14
		JP-A- 62026227	87-02-04